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Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

München, den 14. April 2003
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Der Präsident
Im Auftrag

Agurks

Method and devices for DNA methylation analysis

Field of the invention

5   The analysis of methylation of genomic DNA is proving to be of increasing importance. Aberrant genomic methylation patterns have been shown to be implicated in a wide range of disease conditions, including cancer. DNA methylation analysis requires the development of a range of tools

10   specific to the detection of DNA methylation as conventional techniques such as PCR and sequencing are not capable of distinguishing 5-methyl cytosine from unmethylated cytosine.

15   ## Prior Art

The most common covalent modification of genomic DNA is the methylation of cytosine to 5 methyl cytosine. In eukaryotic cell systems the bulk of methylation activity takes place during the S phase of the cell cycle. Complex

20   tissue specific methylation patterns established during development are preserved in newly replicated DNA by the action of maintenance methyltransferases. These enzymes act to methylate genomic DNA that has been semiconservatively replicated. The methyl transfer reaction proceeds

25   through a non specific binding of the transferase to the hemimethylated DNA strand, identification of the target base followed by the recruitment of the methyl donor group, most commonly S-adenosyl-L-methionine (AdoMet) to the active site. DNA methyltransferases (m5C Mtase) at-

30   tach a methyl group to the 5 position carbon. The reaction is carried out via a covalent intermediate between the enzyme and the base whereby the target cytosine is flipped through 180 degrees. The mechanism of methyltransferase dependant cytosine methylation is further re-

35   viewed in articles such as Cheng and Roberts 'AdoMet-

dependant methylation, DNA methyltransferases and base flipping' Nucleic Acids Res. 15;29(18):3784-95.

Several species of methyltransferases have been identi-
fied, of particular interest to this invention are the
family of maintenance methyltransferases that propagate
the methylation pattern of hemimethylated DNA within the
unmethylated strand, such as Dnmt1. The in vitro action
mechanism of DNMT1 is fully discussed in Pradhan, S., Ba-
colla, A., Wells, R. D., Roberts, R. J. . 'Recombinant
Human DNA (Cytosine-5) Methyltransferase. I. Expression,
Purification and comparison of de novo and maintenance
methylation.' J. Biol. Chem. 274: 33002-33010 and Bacolla
A, Pradhan S, Roberts RJ, Wells RD.'Recombinant human DNA
(cytosine-5) methyltransferase. II. Steady-state kinetics
reveal allosteric activation by methylated DNA' J Biol
Chem. 12;274(46):33011-9.

Cytosine methylation plays an important role in gene ex-
pression and regulation and has been shown to be critical
in the maintenance of normal cellular functions. It is
associated with genomic imprinting, embryonic development
and a wide variety of diseases, including cancer.

For example, aberrant DNA methylation within CpG islands
is common in human malignancies leading to abrogation or
overexpression of a broad spectrum of genes (Jones, P.A.
Cancer Res 65:2463-2467, 1996). Abnormal methylation has
also been shown to occur in CpG rich regulatory elements
in intronic and coding parts of genes for certain tumours
(Chan, M.F., et al., Curr Top Microbiol Immunol 249:75-
86,2000). Using restriction landmark genomic scanning,
Costello and coworkers were able to show that methylation
patterns are tumour-type specific (Costello, J. F., et
al., Nat Genet 24:132-138, 2000). Highly characteristic
DNA methylation patterns could also be shown for breast

cancer cell lines (Huang, T. H.-M., et al., Hum Mol Genet
8:459-470, 1999). Genome wide assessment of methylation
status represents a molecular fingerprint of cancer tis-
sues.

5

Therefore it follows that the analysis of methylation
patterns within genomic DNA is of considerable impor-
tance. However, 5-methylcytosine analysis currently can-
not be carried out using standard molecular biological
10 tool. 5-methylcytosine has the same base pairing behav-
iour as unmethylated cytosine therefore it cannot be
identified by sequencing. Moreover, the epigenetic infor-
mation carried by 5-methylcytosine is completely lost
during PCR amplification. Current methods of analysis are
15 generally carried out using one of two methods. Firstly,
methylation sensitive restriction enzyme digest, and sec-
ondly by the more versatile technique of bisulphite
treatment followed by PCR analysis.

20 Bisuphite treatment followed by alkaline hydrolysis con-
verts cytosine within a nucleic acid sample to uracil.
The treatment is highly specific in that 5-methylcytosine
remains unconverted. Thus PCR amplification of the
treated DNA results in the synthesis of amplificate nu-
cleic acids wherein thymine is substituted for unmethy-
lated cytosine within the original genomic sequence. The
bisulphite treatment is often carried out on minute quan-
tities of genomic DNA which may be lost during handling.
The sensitivity of the technique is improved by use of an
30 agarose matrix within which the DNA is enclosed thus pre-
venting the diffusion and renaturation of the DNA (bisul-
fite only reacts with single-stranded DNA), and which re-
places all precipitation and purification steps with fast
dialysis (Olek A, Oswald J, Walter J. A modified and im-
35 proved method for bisulphite based cytosine methylation
analysis. Nucleic Acids Res. 1996 Dec 15;24(24):5064-6).

In practice the utility of the bisulphite treatment is often limited by the sensitivity of the technique to small samples. Furthermore, the requirement of the agarose step in order to limit DNA loss reduces the suitability of the technique to automization.

Methods for the amplification of specific DNA targets are based upon template directed primer extension by polymerases. The most widely utilised of these methods is the polymerase chain reaction 'PCR' (Mullis, K. et al., Cold Spring Harbor Symp. Quant. Biol. 51:263-273 (1986); Erlich H. et al., EP 50,424; EP 84,796, EP 258,017, EP 237,362; Mullis, K., EP 201,184; Mullis K. et al., U.S. Pat. No. 4,683,202; Erlich, H., U.S. Pat. No. 4,582,788; Saiki, R. et al., U.S. Pat. No. 4,683,194 and Higuchi, R."PCR Technology," Ehrlich, H. (ed.), Stockton Press, NY, 1989, pp 61-68).

In the polymerase chain reaction successive cycles of denaturation are followed by annealing and polymerisation. In the first step the DNA double helix is denatured by transient heating. This is followed by the annealing of two species of primers, one to each strand of DNA. Subsequently the annealed primers are extended using a polymerase. This is followed by the denaturation of the resultant double stranded nucleic acids, allowing each strand to serve as a template for another cycle of template directed primer extension.

The polymerase chain reaction (hereafter PCR) is most commonly performed in disposable reaction tubes such as small, plastic microcentrifuge tubes or test tubes which are placed in an instrument containing a thermally controlled heat exchanger. Examples of these instruments are disclosed in U.S. Pat. No. 5,038,852, U.S. application Ser. No. 07/709,374, filed Jun. 3, 1991, and U.S. appli-

cation Ser. No. 07/871,264, filed Apr. 20, 1992. Alterna-
tive devices for the PCR analysis of nucleic acids
wherein the reaction is carried out in capillary tubes
have been described in U.S. Pat. No. 5,779,977 .

5

The heat exchanger in these instruments is typically a
metal block; however, hot air ovens and water baths also
have been used. The temperature of the reaction mixture
in the reaction tubes is changed in a cyclical fashion to
10   cause denaturation, annealing and extension reactions to
occur in the mixture. Three separate incubation tempera-
tures commonly were used in the first generation PCR
thermal cycling applications. These were typically around
94.degree. C. for denaturation, around 55.degree. C. for
15   annealing, and around 72.degree. C. for extension. More
recently, the annealing and extension incubations have
frequently been combined to yield a two temperature incu-
bation process, typically around 94.degree. C. for dena-
turation, and around 50.degree.-65.degree. C. for an an-
20   nealing and extension incubation. The optimal incubation
temperatures and times differ, however, with different
targets and primer oligonucleotides.

Description

25   The invention relates to a method and devices for the en-
zymatic amplification of nucleic acids whereby the methy-
lation pattern of said nucleic acid is conserved in the
amplificate sequence. The method presents improvements
over the basic polymerase chain reaction in that a fur-
30   ther methylation step is carried out thereby conserving
the complex methylation pattern of a genomic DNA in the
amplificate nucleic acids. Subsequent to each cycle of
the polymerase chain reaction, the hemimethylated nucleic
acid is contacted with a maintenance methyltransferase
35   thereby allowing for the methylation of the unmethylated
strand of the nucleic acid. Methylation of the hemimethy-

lated DNA by a maintenance methyltransferase is such that the specific methylation pattern of the CpG dinucleotides within the template strand of the nucleic acid is replicated in the unmethylated strand. The described invention thereby allows for the preservation of complex genomic methylation patterns within amplificate nucleic acids.

The maintenance of complex methylation patterns within nucleic acid amplificates allows for the analysis of samples using a variety of methylation specific techniques. Such techniques, which include bisulphite analysis and methylation sensitive restriction enzyme digest were heretofore carried out using limited amounts of DNA samples and/or in combination with a polymerase chain reaction.

Definitions

The following terms and phrases as used herein are intended to have the meanings as described below.

In the context of the present invention the term 'methylation pattern' is taken to mean the specific consecution of 5 methyl cytosine within a nucleic acid sequence.

In the context of the present invention the term 'template nucleic acid' refers to a single stranded nucleic acid which serves as a template for the synthesis of a nucleic acid from a primer oligonucleotide.

In the context of the present invention the term 'synthesised nucleic acid' is taken to mean a nucleic acid which is the product of a template directed primer extension reaction.

The invention comprises method and devices for the methylation pattern retaining enzymatic amplification of nu-

cleic acids. The method comprises a modified form of the
polymerase chain reaction, wherein an additional methyla-
tion step during each cycle of the reaction allows for
the replication of complex methylation patterns present
within a genomic DNA sample in amplificate nucleic acids.
The method comprises four steps of which the first three
steps are known in the art, for example, in US patents
numbers 4,683,195, 4,683,202, and 4,800,159. The fourth
step is the novel methylation retaining step.

It is one object of the present invention to provide a
method for the amplification of genomic DNA whereby the
cytosine methylation pattern of the genomic DNA is re-
tained in the amplificate sequence(s), said method com-
prising the following steps:
(a) heating the genomic DNA to a temperature operative to
cause denaturation
(b) cooling the denatured DNA in the presence of single
stranded oligonucleotide primers such that the primers
anneal to the DNA
(c) heating the mixture in the presence of a polymerase
and nucleotides to a temperature such that the primers
are extended
(d) contacting the double stranded nucleic acid with a
methytransferase and a methyl donor molecule under condi-
tions conducive to the methylation of the synthesised
strand such that the CpG dinucleotides within the synthe-
sised strand are methylated according to the methylation
status of the corresponding CpG dinucleotide on the tem-
plate strand thereby preserving the genomic methylation
pattern
(e) repeating steps A-D a desired number of times to
reach a desired number of nucleic acids.

In a preferred embodiment of the present invention the
methytransferase is a maintenance methyltransferase. In

a further preferred embodiment the methytransferase is
DNA (cytosine-5) Methyltransferase (DNMT 1).

According to the invention it is preferred that the
5   methyl donor molecule is S-adenosylmethionine.

It is also preferred according to the invention that the
methyl group carries a detectable label which is incorpo-
rated into the synthesised nucleic acid strand.
10

In a further preferred embodiment of the present inven-
tion that a plurality of primer oligonucleotides are im-
mobilised on a solid surface.

15   It is also preferred that the methyltransferase is immo-
bilised on a solid surface.

According to the invention it is also preferred that the
polymerase is immobilised on a solid surface.
20

In another preferred embodiment of the present invention
a further step (f) is present that is a treatment with an
agent capable of distinguishing between methylated and
unmethylated cytosine bases. Hereby it is preferred that
the agent is a methylation sensitive restriction enzyme.
It is especially preferred according to the invention
that the agent is a bisulphite solution.

Another object of the invention is a device for the me-
30   thylation pattern retaining amplification of nucleic ac-
ids according to Claim 1 said device comprising two or
more reaction chambers, channel means providing fluid
connections between adjacent chambers and the first and
last reaction chambers, temperature regulating means for
35   controlling the temperature of each reaction chamber.

It is preferred according top the invention that the device for the methylation pattern retaining amplification of nucleic acids comprises two vessels, a reaction chamber, temperature regulating means for controlling the temperature of the reaction chamber, means for transferring liquid reagent form the first and second vessels to the reaction chamber, channel means providing fluid connections between adjacent chambers and the first and last reaction chambers means for draining liquid reagents from the reaction chamber.

A nucleic acid obtained according to the method of the invention by optionally using the device according to the invention.

A furtjer object of the present invention is a method of manufacturing a methylated nucleic acid according to the method of the invention by optionally using the device according to the invention.

The details of method of the present invention are as follows.

In the first step of the method (hereinafter referred to as Step A) the sample DNA is heat denatured allowing the single stranded DNA to be used in the analysis, suitable melting temperatures are dependant upon several variables including GC content of the sequence and length of sequence, but in general may be 95°C or higher for 15 seconds to 2 minutes. In the second step (hereinafter referred to as Step B) oligonucleotide primers are annealed to the template sequence at a lower temperature, (typically between 40°C and 60°C for 30 to 60 seconds), again dependant upon the GC content and length of the primers. The oligonucleotides are able tó form stable associations ('anneal') with the single stranded DNA (hereinafter re-

ferred to as the template strand) and thus serve as prim-
ers for nucleic acid synthesis by a DNA polymerase. In
the third step (hereinafter referred to as Step C) a cor-
responding nucleic acid strand to the template is synthe-
5   sised from the primer oligonucleotide by means of the po-
lymerase and deoxynucleotide triphosphates (dNTPs). The
temperature is raised to an optimum for the polymerase,
which in the case of commonly used thermostable poly-
merases is approximately 74°C, primer extension then
10   lasts approximately 1 to 2 minutes. These steps are all
common to the polymerase chain reaction. Reactions take
place in a mixture which includes a sample of the target
DNA, a thermostable DNA polymerase, oligonucleotide prim-
ers, deoxynucleotide triphosphates (dNTPs), reaction
15   buffer, magnesium and optional additives. Reaction tem-
peratures and times must be optimised according to fac-
tors including, for example, GC content and length of se-
quence and primers.

20   Subsequent to enzymatic amplification, the resultant he-
mimethylated nucleic acid is enzymatically methylated
according to the methylation status of the CpG dinucleo-
tides within the template strand. The enzyme should be a
maintenance methyltransferase with an affinity for hemi-
methylated DNA .

This step is executed a user defined number of times.
Each repetition of Steps A-D results in a doubling of the
number of nucleic acid molecules within the sample. The
30   exponential increase in the quantity of synthesised nu-
cleic acid enables the production of sufficient quanti-
ties of methylated nucleic acids for use in other methy-
lation specific analysis techniques such as methylation
sensitive restriction enzyme analysis and bisulphite
35   treatment with a greatly increased efficiency.

Suitable methylation enzymes for use in Step D of the
method are limited to those capable of methylating the
cytosine at the 5 position according to the methylation
status of the cytosine within the corresponding CpG dinu-

5   cleotide on the template strand. In the case of a cyto-
sine within a CpG upon the template strand being methy-
lated, then the corresponding CpG to which it is hybrid-
ised on the synthesised strand will be methylated by ac-
tion of the enzyme at the 5 position of the cytosine

10   base. If the cytosine within said CpG is unmethylated
then the corresponding CpG on the synthesised strand will
remain unmethylated. The reaction is carried out using
appropriate buffers and other reagents and reaction con-
ditions as recommended by the supplier of the enzyme,

15   this may include a methyl donor molecule such as, but not
limited to S-adenosylmethionine. Furthermore said methyl
group may carry a detectable label, for example a fluo-
rescent label.

20   The enzyme may be from any source e.g. Human, Mouse, re-
combinant. In a preferred embodiment the enzyme is DNA
Methyltransferase 1 (DNMT1). In a further preferred em-
bodiment the methyltransferase is immobilised upon a
solid surface.

The invention further relates to devices for the methyla-
tion retaining amplification of a DNA sequence. Several
embodiments of devices for the methylation retaining PCR
of nucleic acids are envisioned. All devices described

30   herein are controlled by a user defined protocol imple-
mented by a programmable computer. The computer is capa-
ble of controlling all variables of the system including
sample handling, flow, velocity, pressure, and tempera-
ture. Differences between said embodiments arise due to

35   the different means of heating and cooling the reaction

mixture and also the alternative means employed for con-
tacting the reagents.

Three embodiments of such devices are described below.

5

Multiple reaction chamber methylation retaining PCR de-
vice

A first embodiment of a methylation retaining PCR device
is illustrated in Figure 1, said embodiment is hereinaf-
10   ter referred to as Device 1. The illustrated embodiment
has four thermostatted units. Each unit comprises a means
for temperature maintenance (1), reaction chamber for
containing the reaction solution (2), said reaction cham-
bers connected by tube means (3) for transporting the re-
15   action solution between the reaction chambers and further
comprising means for introducing additional reagents into
the reaction solution. Two of the reaction chambers may
further each comprise a solid support (4) upon which is
immobilised the enzymatic means for carrying out Steps C
20   or D of the reaction respectively. In a further preferred
embodiment the reaction chamber within which Step C is
carried out does not further comprise a solid surface up
on which the polymerase is immobilised, the polymerase is
a component of the reaction solution. The reaction cham-
bers are each connected by tube means in order to trans-
port the reaction solution between each chamber. Trans-
port through the tubes is carried out by means of a
pump(6) , preferably a peristaltic pump. The tubes fur-
ther comprise valves ensuring that the reaction solution
30   flows in a unidirectional manner. In a further preferred
embodiment the pump mechanism may be a plunger and seal
arrangement, similar to a syringe. Said pump may be con-
nected to a first reaction chamber within which the ini-
tial denaturation reaction takes placer. The pump mecha-
35   nism is under control of a computer programmed to carry
out the PCR protocol. The pump or plunger is activated

back and forth to draw reaction mixture into the reaction chamber,

In a first reaction chamber, Step A of the reaction is carried out whereby the methylated nucleic acid sample is heat denatured in a reaction solution further containing necessary buffers and reagents. Said reaction chamber is kept at a consistent suitable temperature, generally between 85°C and 95°C. The reaction mixture is then passed to the second reaction chamber in order to carry out Step B of the reaction. The second reaction chamber is kept at a consistent temperature suitable for the annealing of primer oligonucleotides to the target DNA. Said temperature is dependant upon the composition of the primers but a general estimate may be calculated using the formula $Tm = 81.5 + 16.6 \times (\log_{10}[Na+]) + 0.41 * (\%G+C) - 675/n$ wherein [Na+] is the molar salt concentration; [K+] = [Na+] and n = number of bases in the oligonucleotide .Step B according to Claim 1 is carried out in the second chamber.

Upon annealing of the primer oligonucleotides the reaction mixture is passed to the third reaction chamber whose temperature is regulated to that suitable for the action of the polymerase used in the method. In the case of the commonly used Taq polymerase this would be 74°C.

As taught by Kim and Smithies (Nucleic Acids Research 16, 8887-8903) incubation at the intermediate "extension" temperature is unnecessary. Accordingly only two temperatures are required one in the range from 37-72.degree. C. for annealing and extension and one in the 85-98.degree. C. range for denaturation. Therefore, a further embodiment of Device 1 may consist of only three chambers wherein chambers 2 and 3 are replaced by a single cham-

ber, said chamber further comprising a solid support upon which the polymerase is immobilised.

Subsequent to primer extension the reaction mixture is passed to a fourth reaction chamber for Step D. Said reaction chamber further comprises a solid phase upon which a methyltransferase is immobilised. At this stage the required co factor for the methyltransferase (e.g. S-adenosylmethionine) is added to the reaction solution. The reaction mixture is passed through the reaction chamber such that the hemimethylated double stranded nucleic acids are contacted with the methyltransferase enzyme sufficient for the methylation of all nucleic acids within the reaction mixture. Reaction times and conditions are calculated and optimised according to the methyltransferase as well as CpG composition of the amplificate nucleic acids. The reaction solution is then passed back to the first reaction chamber allowing Steps A-D to be repeated until the desired quantity of amplificates is synthesised.

In its simplest form, the thermostatic means described in the inventions above may comprise two metal block heat exchangers separated by a layer of insulation. The heat exchangers could also be other types of heat exchangers such as thermostatically controlled constant temperature fluid baths. Each metal block heat exchanger is preferably made of aluminium or some other good heat conducting metal to minimise temperature gradients therein.

The temperature of each metal block is maintained at a constant temperature by any suitable temperature control system. A suitably programmable control system which may be used is disclosed in U.S. Pat. No. 5,038,852 and in U.S. Patent application No. 07/871,264, filed Apr. 20, 1992.

Peltier devices are ideal for controlling the temperatures of the metal blocks because these metal block heat exchangers are each maintained at a constant temperature.

5   Suitable known temperature sensing and feedback control circuits (not shown) are necessary to control the direction of current flow through the Peltier devices to maintain the block temperature constant by extracting heat from the block when it gets too hot and adding heat when

10   it gets too cold. Any other temperature control system will also work for the blocks such as resistance heaters and/or heated/chilled fluid circulating through passages in the metal blocks with the chilled fluid being, for example, tap water or antifreeze chilled by circulating

15   freon of a refrigeration unit, depending upon the desired temperatures of the blocks.

Sample handling and reaction conditions, including, flow, velocity, pressure, and temperature are all controlled

20   according to a user defined protocol by a computer (5). Connections between said computer and components of the device illustrated by, but not limited to, dotted lines.

Single reaction chamber methylation retaining PCR device

25   In a further embodiment of a methylation retaining PCR device (hereinafter referred to as Device 2), all steps of the method are carried out in one reaction chamber. The device comprises a thermocycling reaction chamber (10) , two vessels (7) containing reagents (8) required

30   for the different stages of the procedure. The reaction chamber is thermostatically cycled between temperatures suitable for Steps A, B C and D of the reaction as described above.

35   In said embodiment the apparatus comprises 2 vessels (7) and a reaction chamber (10). The vessels are each con-

nected to the reaction chamber by tube means (11), reac-
tion solution is passed through the tubes by means of a
pump (12), preferably a peristaltic pump. The tubes fur-
ther comprise valves (9) ensuring that the reaction solu-
tion flows in a unidirectional manner. A first vessel
contains all reagents necessary for Steps A to C accord-
ing to Claim 1 for example, but not limited to, dNTPs,
reaction buffer and polymerase. A second vessel contains
all reagents necessary for Step D for example, but not
limited to; reaction buffer, methyltransferase and co-
factors. The reaction chamber is a thermocycling vessel
comprising a solid surface (13) upon which an excess of
primer oligonucleotides required during Step B of the re-
action are immobilised. Sample DNA and reagents from the
first vessel are passed into the reaction chamber where
the denaturation, annealing and extension processes
(Steps A-C) are carried out. Primer oligonucleotides im-
mobilised upon a solid surface hybridise to a genomic DNA
during Step B of the first cycle of the reaction. Single
stranded nucleic acid molecules generated by Step A of
the reaction hybridise to the primer oligonucleotides.
After extension (Step C), methylation (Step D) and dena-
turation (Step A) the single stranded template nucleic
acids hybridise to unused primer oligonucleotides in the
vicinity of the primer oligonucleotides to which they
were hybridised in the previous cycle.

Upon completion of the extension (Step C) and prior to
the methylation reaction (Step D) the reaction mixture is
drained from the reaction chamber by means of an outlet
(15). Said outlet may further comprise a valve and pump
mechanism (12) as illustrated. Reagents from the second
vessel are passed into the reaction chamber and methyla-
tion of the hemimethylated nucleic acids is carried out
under appropriate conditions. The reaction chamber may
then be drained of reagents allowing reagents from the

first vessel to be passed into the reaction chamber and
allowing Steps A to D of the reaction to proceed again
until the desired quantity of methylated nucleic acids is
synthesised.

5

Sample handling and reaction conditions, including, flow,
velocity, pressure, and temperature are all controlled
according to a user defined protocol by a computer (14).
Connections between said computer and components of the
10   device illustrated by, but not limited to, dotted lines.

In one embodiment of the invention (e.g. Device 1) the
polymerase and methylase enzymes as used in Steps C and D
of the reaction may be immobilised upon a solid support.
15   In a further embodiment (e.g. Device 2) primer oligonu-
cleotides as utilised in Step C of the method are re-
quired to be immobilised upon a solid support. The solid
support may be beads, particles, sheets, dipsticks, rods,
membranes, filters, fibres (e.g., optical and glass), and
20   suchlike. Preferably, the solid support is a bead. The
material composition of the solid support includes, but
is not limited to, polystyrene, nitrocellulose, plastic,
nylon, glass, silica, metal, metal alloy, polyacrylamide,
polyacrylate, crosslinked-dextran and combinations
thereof. Preferably, the solid support is thermally sta-
ble (e.g., able to withstand temperatures of up to
100.degree. C.) to withstand thermocycling conditions as
described in the invention. Preferably, the solid support
is capable of being modified by the attachment of oli-
30   gonucleotide primers. Methods for the immobilisation of
oligonucleotides are known in the art and include the use
of photolabile groups and solid phase chemistry (U.S.
Patent 5,744,305). Methods for the immobilisation of en-
zymes are also well known in the art for example in 'Im-
35   mobilization of Enzymes and Cells' Bickerstaff and Wal-
ker, Humana Press 1996.

Double reaction chamber methylation retaining PCR device

A third embodiment of a methylation retaining PCR device is illustrated in Figure 3, said embodiment is hereinaf-
5   ter referred to as Device 3. The illustrated embodiment of the device has two reaction chambers. A first reaction chamber comprises a thermocycling unit (16) and a vessel (17) for containing the reaction solution. Steps A to C of the reaction are carried out in this chamber. The re-
10   action solution is then pumped from the first reaction chamber to a second reaction chamber (18) using tube means (19) wherein Step D of the method is carried out. Reagents required for Step D of the reaction are con-
tained in a vessel (20) which is connected to reaction
15   chamber (18) by tube means. Reagents from the vessel (20) are pumped into reaction chamber (18) by tube means (21). The pump (22) may take in form standard in the art, for example a peristaltic pump. However, particularly pre-
ferred is a syringe pump.

20

Sample handling and reaction conditions, including, flow, velocity, pressure, and temperature are all controlled according to a user defined protocol by a computer (23). Connections between said computer and components of the
25   device illustrated by, but not limited to, dotted lines.

Thermocycling units suitable for use in nucleic acid am-
plification techniques are known in the art and commer-
cially available, for example, from manufacturers such as
30   Perkins Elmer and Eppendorf.

Figures

Figure 1
35   Figure 1 illustrates a multiple reaction chamber methyla-
tion retaining PCR device.

Figure 2

Figure 2 illustrates a single reaction chamber methyla-
tion retaining PCR device.

5

Figure 3

Figure 3 illustrates a double reaction chamber methyla-
tion retaining PCR device.

10

Examples
Example 1

Genomic DNA commercially available from Promega is used
in the analysis. A CpG rich fragment of the regulatory

15   region of the GSTPi gene is used in the analysis. The DNA
is firstly artificially methylated at all cytosine 5 po-
sitions within the CpGs (upmethylation). The upmethylated
DNA is then amplified using one round of PCR. The resul-
tant amplificate is then divided into two samples, Sample

20   A (the control sample) is amplified using conventional
PCR. Sample B is amplified according to the disclosed
method. The two samples are then compared in order to as-
certain the presence of methylated CpG positions within
Sample B. The comparison is carried out by means of a bi-
sulphite treatment and analysis of the treated nucleic
acids.

Upmethylation

Reagents:

30

DNA

SssI Methylase (concentration 2 units/µl).

SAM (S-adenosylmethionine)

4,5 µl Mssl-Buffer (NEB Buffer B+ (10 mM Tris-HCl 300 mM
NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol,
500 µg/ml BSA, 50% glycerol (pH 7.4 at 25°C)pH 7.5; 10 mM
5      $MgCl_2$; 0,1 mg/ml BSA)

dd water (0.2 µm-filtered autoclaved, DNases, RNases,
proteases, phosphatases-free).

.10   Method:
Reagents are combined and incubated at 37 degrees for 16
hours. The sample may then be stored in the refrigerator
(+4°C).

15   The upmethylated DNA is digested using the restriction
enzyme

PCR
        Reagents:
20   primer I : TTCGCTGGAGTTTCGCC

primer II :GCTTGGGGGAATAGGGAG

HotStart Taq Polymerase (QIAGEN)
25   10 x PCR buffer (QIAGEN)
dNTP solution (25 mM each)
water (0.2 µm-filtered, autoclaved, DNases, RNases, pro-
teases, phosphatases-free).

30   Reagents are to be combined in a reaction solution in the
order above. The reaction solution is then cycled in a
thermalcycler according to the following. An initial de-
naturation at 95°C is carried out for 15 min. This is
followed by primer annealing at 55°C for 45 sec. and
35   elongation at 72°C for 1.5 min.

The resultant reaction solution is then divided into two equal samples, A and B. Each sample is treated as below.

Sample A

5 Standard PCR as described above. The reaction is cycled for 40 cycles at 95°C for 1 minute, 55°C for 45 sec. and elongation at 72°C for 1.5 min.

Sample B

10 Reagents:

Human DNA (cytosine-5) Methyltransferase (New England Biolabs)

Dnmt 1 reaction buffer (50 mM TrisHCL pH7.8, 1 mM EDTA, 1 MM dithiothreitol, 7 µg/ml PMSF, 5% glycerol)

15 100 µg/ml BSA

Steps 1 to 4 are repeated 40 times:

1. DNA is precipitated and pelleted, resuspended using
20 Dnmt1 reaction buffer, DNMT and BSA .
2. The reaction solution is incubated at 37°C.
3. DNA is precipitated and pelleted, resuspended using PCR reagents as above.
4. One cycle of PCR is carried out at 95°C for 1 minutes,
25 55°C for 45 sec. and elongation at 65°C for 2 min.

Sample Analysis

Both samples are analysed in order to ascertain their
30 relative levels of methylation. In a first step the two samples are treated in order to distinguish between me-thylated and non methylated cytosines. The treatment is carried out using a solution of sodium-disulfite. The treatment converts cytosine to thymine while preserving
35 5-methyl-cytosine as cytosine. Sample A is thereby thymine rich relative to Sample B, which is relatively

cytosine rich. Following bisulphite treatment both samples are analysed by means of sequencing in order to ascertain their degree of methylation (i.e. relative concentrations of cytosine and thymine). Sequencing is carried out by means of the Sanger method using the ABI 310 sequencer (Applied Biosystems).

Claims

1. A method for the amplification of genomic DNA whereby the cytosine methylation pattern of the genomic DNA is retained in the amplificate sequence(s), said method comprising the following steps:

   (a) heating the genomic DNA to a temperature operative to cause denaturation

   (b) cooling the denatured DNA in the presence of single stranded oligonucleotide primers such that the primers anneal to the DNA

   (c) heating the mixture in the presence of a polymerase and nucleotides to a temperature such that the primers are extended

   (d) contacting the double stranded nucleic acid with a methytransferase and a methyl donor molecule under conditions conducive to the methylation of the synthesised strand such that the CpG dinucleotides within the synthesised strand are methylated according to the methylation status of the corresponding CpG dinucleotide on the template strand thereby preserving the genomic methylation pattern

   (e) repeating steps A-D a desired number of times to reach a desired number of nucleic acids.

2. A method according to Claim 1 wherein the methytransferase is a maintenance methyltransferase.

3. A method according to Claim 1 wherein the methytransferase is DNA (cytosine-5) Methyltransferase (DNMT 1).

4. A method according to Claims 1 to 3 wherein the methyl donor molecule is S-adenosylmethionine.

5. A method according to Claims 1 to 4 wherein the methyl group carries a detectable label which is incorporated into the synthesised nucleic acid strand.

6. A method according to Claims 1 to 5 wherein a plurality of primer oligonucleotides are immobilised on a solid surface.

7. A method according to Claims 1 to 5 wherein the methyltransferase is immobilised on a solid surface.

8. A method according to Claims 1 to 5 wherein the polymerase is immobilised on a solid surface.

9. A method according to Claim 1 further comprising Step (f) a treatment with an agent capable of distinguishing between methylated and unmethylated cytosine bases.

10. A method according to Claim 9 wherein the agent is a methylation sensitive restriction enzyme.

11. A method according to Claim 9 wherein the agent is a bisulphite solution.

12. A device for the methylation pattern retaining amplification of nucleic acids according to Claim 1 said device comprising two or more reaction chambers, channel means providing fluid connections between adjacent chambers and the first and last reaction chambers, temperature regulating means for controlling the temperature of each reaction chamber.

13. A device for the methylation pattern retaining amplification of nucleic acids according to Claim 1 to 6 comprising

two vessels,

a reaction chamber,

temperature regulating means for controlling the temperature of the reaction chamber,

5   means for transferring liquid reagent form the first and second vessels to the reaction chamber,

channel means providing fluid connections between adjacent chambers and the first and last reaction chambers

10   means for draining liquid reagents from the reaction chamber.

14. A nucleic acid obtainable by a method according to one of the claims 1 to 11.

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15. A method of manufacturing a methylated nucleic acid using a method according to one of the claims 1 to 11.

Abstract

The invention outlines a method for the methylation pat-
5   tern retaining amplification of nucleic acid molecules.
Furthermore the invention describes several devices for
use in the methylation pattern retaining amplification of
nucleic acid molecules.

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Fig.1

Fig. 2

Fig. 3